Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions

Heath A. MacMillan,1 Jonas L. Andersen,1 Volker Loeschcke,2 and Johannes Overgaard1

1Zoophysiology, Department of Bioscience, Aarhus University, Aarhus C, Denmark; and 2Genetics, Ecology and Evolution, Department of Bioscience, Aarhus University, Aarhus C, Denmark

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MacMillan HA, Andersen JL, Loeschcke V, Overgaard J. Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions. Am J Physiol Regul Integr Comp Physiol 308: R823–R831, 2015. First published March 11, 2015; doi:10.1152/ajpregu.00465.2014.—Many insects, including the model holometabolous insect *Drosophila melanogaster*, display remarkable plasticity in chill tolerance in response to the thermal environment experienced during development or as adults. At low temperatures, many insects lose the ability to regulate Na+ balance, which is suggested to cause a secondary loss of hemolymph water to the tissues and gut lumen that concentrates the K+ remaining in the hemolymph. The resultant increase in extracellular [K+] inhibits neuromuscular excitability and is proposed to cause cellular apoptosis and injury. The present study investigates whether and how variation in chill tolerance induced through developmental and adult cold acclimation is associated with changes in Na+, water, and K+ balance. Developmental and adult cold acclimation improved the chilling tolerance of *D. melanogaster* in an additive manner. In agreement with the proposed model, these effects were intimately related to differences in Na+ distribution prior to cold exposure, such that chill-tolerant flies had low hemolymph [Na+], while intracellular [Na+] was similar among treatment groups. The low hemolymph Na+ of cold-acclimated flies allowed them to maintain hemolymph volume, prevent hyperkalemia, and avoid injury following chronic cold exposure. These findings extend earlier observations of hemolymph volume disruption during cold exposure to the most ubiquitous model insect (*D. melanogaster*), highlight shared mechanisms of developmental and adult thermal plasticity and provide strong support for ionoregulatory failure as a central mechanism of insect chill susceptibility.

chill susceptible; fruit fly; chilling injury; ion balance; phenotypic plasticity

MANY INSECTS LOSE NEUROMUSCULAR function and enter chill coma (a state of complete paralysis) at a low temperature termed the critical thermal minimum (CTmin) (42, 45). Some of these species have evolved the ability to tolerate or prevent ice formation and can survive exposure to subzero temperatures that cause chill coma in a partially frozen or freeze-avoidant state (5, 61). However, the majority of insect species are chill-susceptible, meaning they succumb to physiological effects of chilling at temperatures above those that cause freezing (6). With time spent at or below the CTmin, many chill-sensitive insects accumulate injuries (termed chilling injury) and ultimately die (3, 34, 43). If the chilling is mild, however, insects can recover neuromuscular function, and the time required to recover the ability to stand following low-temperature exposure is termed chill coma recovery time (CCRT) (16). Chilling tolerance is closely correlated to the poleward distribution limits of insects (1, 3, 21–23, 32, 50), and consequently, the CTmin, CCRT, and incidence of chilling injury or death are often used to build and test theory of biogeography in a changing climate (9, 50, 59).

The vast majority of *Drosophila* species overwinter as adults, and the overwintering success of an individual drosophilid is, thus, dependent on its ability to acquire cold tolerance during ontogeny (3, 25, 30). Consequently, insects with a cosmopolitan distribution, like the model insect *Drosophila melanogaster*, have evolved an impressive capacity to alter their cold tolerance in response to prior low-temperature exposure. The chilling tolerance of *D. melanogaster*, for example, can be altered in response to changes in the temperature at which development occurs (developmental acclimation), as well as the temperatures experienced by mature adults (14, 26, 55, 56). Thermal plasticity of adult insects is typically categorized by the duration of preexposure to a nonlethal low temperature; long-term responses (i.e., on the order of days) are typically termed thermal acclimation, while improvements in thermal performance induced over a much shorter time period (minutes to hours) are termed hardening (37, 62). In this study, we focus on the individual and interactive effects of developmental and long-term adult acclimation and investigate how these treatments relate to whole-organism measures of cold tolerance and ion balance that are hypothesized to underlie insect chill tolerance (18, 34, 35, 40, 42, 67).

When exposed to the cold, the muscle fibers of *Drosophila* and other insects rapidly depolarize, and the insect enters chill coma (27, 40, 64). What causes this initial depolarization is under active investigation, but an increase in the membrane permeability of Na+ relative to K+ or a reduction in the activity of electrogenic ion-motive pumps relative to membrane resistance have been suggested (18, 27, 40, 64). In all chill-susceptible insects studied to date, prolonged exposure to low temperature causes a progressive increase in extracellular [K+], which correlates with the development of chilling injury and mortality following cold exposure (2, 17, 34, 35, 39, 40, 43). Cell membrane potential is heavily dependent on the maintenance of low extracellular [K+] (4, 19, 28, 29, 58, 64), so rising extracellular [K+] is thought to render the neuromuscular system excitable through further cell membrane depolarization (4, 40). The extent of [K+] disruption following prolonged cold exposure and rate at which low hemolymph [K+] is reestablished upon rewarming are closely related to the ability to recover from chill coma (2, 17, 40, 44). Thus, the CTmin, CCRT, and chilling injury all appear to be tied to the effects of temperature on ion and water distribution, ion permeability, and the resting membrane potential of excitatory tissues. Although the specific mechanisms underlying these different...
measures of cold tolerance may differ (18, 40), it is clear that maintenance of water and ion homeostasis is vital for insect cold resistance. As observed in other insects, extracellular [K⁺] increases during chilling in D. melanogaster (39), but what causes this increase has not been confirmed in any holometabolous insect. In hemimetabolous insects, the concentration increase appears to have little to do with a failure of K⁺ transport per se, but is instead caused by a loss of Na⁺ and water balance (43). Na⁺ is a dominant extracellular cation and osmolyte in many insects, and in both crickets and migratory locusts, cold exposure causes a net leak of Na⁺ ions from the hemolymph to the gut lumen (and other tissues in locusts) that contributes to a concurrent loss of hemolymph water (43). This failure of Na⁺ balance is likely driven by a chilling-induced suppression of primary and secondary ion transport processes relative to the passive flux of Na⁺ down its electrochemical gradient (42). Regardless, the resultant shift in water distribution appears to be largely responsible for concentrating the K⁺ remaining in the hemolymph, which ultimately causes cellular depolarization (2, 34, 43) (Fig. 1A).

If cold exposure causes a loss of ion and water balance that leads to injury and death, insects may improve their chilling tolerance by either avoiding the loss of balance, tolerating the loss of balance, and/or more rapidly restoring balance upon rewarming (8, 17). Freeze-avoiding and chill-susceptible insects that have been acclimated to low temperatures as adults all appear to improve their chilling tolerance by avoiding the loss of ion and water balance experienced by their warm-acclimated conspecifics (13, 34, 35, 39). If Na⁺ balance is the primary driver of the ionic and osmotic failure at low temperatures, insects could adopt one or both of two physiological strategies to improve chilling tolerance: 1) maintain Na⁺ distribution at low temperatures by reducing the permeability of membranes and epithelia to Na⁺ and/or increasing primary ion transport to maintain ion balance in the cold and 2) reduce the dependency of water balance on Na⁺ distribution by reducing the electrochemical gradient that drives Na⁺ leak (Fig. 1B). In support of the second strategy, adults of the Drosophila species with low extracellular [Na⁺] have a lower CTₘᵟᵦᵦ than those that maintain high hemolymph [Na⁺], and cold-acclimated D. melanogaster adults similarly maintain low hemolymph [Na⁺] (39).

Developmental temperature and adult acclimation have been demonstrated to have additive effects on cold tolerance in D. melanogaster (14, 56) and so may either operate through different physiological mechanisms, or through additive effects on a shared mechanism of chilling tolerance. We hypothesize that improvements in insect chilling tolerance achieved through cold acclimation, regardless of the life stage in which low temperature is experienced, are driven by reductions in the Na⁺ concentration ratio between the extracellular fluid (hemolymph) and the intracellular fluid or the fluid in the gut lumen. We further hypothesize that these reduced Na⁺ gradients reduce the reliance of water balance on Na⁺ balance, and, thereby, improve chill tolerance by allowing flies to maintain water and ion homeostasis at low temperatures. To test these hypotheses, we induced variation in chill tolerance in D. melanogaster using two forms of phenotypic plasticity (developmental and adult cold acclimation) by rearing and acclimatizing flies at 15°C and 25°C in a fully crossed design (Fig. 2).
MATERIALS AND METHODS

Origin population and thermal acclimation. The stock of *D. melanogaster* used was caught in October of 2010 in Odder, Denmark (55.945°N, 10.213°E), and established from ~550 isofemale lines (60). A laboratory population was maintained in 250-ml plastic bottles containing 50 ml of Leeds *Drosophila* medium (oatmeal-sugar-yeast-agar) at a constant temperature of 25°C at 12:12-h light-dark (L:D) cycle. Groups of ~20 parental flies were allowed 24 h to lay eggs on spoons filled with ~2 ml of medium, and eggs were counted into vials containing 7 ml of medium at controlled density (45 eggs per vial). Eggs collected from the spoons were divided randomly and placed at one of two temperatures (15°C and 25°C) for the duration of their development with the same light cycle (12:12-h L:D). Adult flies were collected upon the day of emergence and randomly divided again for adult acclimation to either 15°C or 25°C, resulting in a fully crossed experimental design of developmental and adult acclimation temperatures (Fig. 2). Flies were separated by sex under light CO₂ anesthesia (less than 5 min) after 3 days of adult acclimation. Males were discarded, and females were counted into fresh vials (10 flies per vial) and placed back at their acclimation temperature for a further 3 days to recover from the CO₂ exposure (15, 48) and to further acclimate. All of the female flies were thus 6 days postemergence, when used for experiments.

Chill coma onset, chill coma recovery time, and lethal time at ~2°C. Chill coma onset temperature was determined as described previously (3, 51). Flies were individually placed in sealed 5-ml glass vials (n = 20 per developmental × adult acclimation group). The vials were placed in a rack that was submerged in a transparent bath containing a mixture of ethylene glycol and water (1:1 vol/vol) preset to 20°C (a temperature between the two experimental conditions). The bath temperature was held at 20°C for 15 min before being decreased at a rate of 0.1°C/min. The flies were continuously observed and those that stopped spontaneous movement were stimulated to move by tapping on the vial. The temperature for onset of chill coma (CTₘᵟₙ) was recorded as the temperature at which a fly elicited no movement in response to stimulation.

CCRT was measured following 6 h at 0°C following previously described methods (3). Briefly, individual flies (n = 20 per developmental × adult acclimation group) were placed in sealed 5-ml vials that were submerged in a mix of ice and water. After 6 h, the vials were removed to room temperature (23°C), and the time required for each fly to spontaneously regain a standing posture was recorded.

The ability of flies to survive a chronic cold exposure was assessed after exposing flies to ~2°C for varying lengths of time. Vials (25 ml), each containing 10 flies, were placed in a water bath containing a mixture of ethylene glycol and water (1:1 vol/vol) that was held at ~2°C. The vials were closed with a foam stopper to allow exchange of respiratory gases. For each group of developmental × adult acclimation, we assayed the survival from five replicates at each experimental time point. Pilot survival tests were completed to determine cold exposure durations that spanned 0 to 100% survival for each experimental group. As a result, flies acclimated as adults to 25°C (irrespective of developmental temperature) were exposed to 2–24 h at ~2°C, while those acclimated as adults to 15°C were given 6–84 h at ~2°C. At the end of the cold exposure, the flies were transferred into vials containing 4 ml of fresh food medium and returned to their respective adult acclimation temperature to recover. After 24 h, survival was assessed by tapping each vial against a table surface, and observing the flies for 10 s. Flies that were able to stand and/or climb were scored as alive, while those that were unable to stand or showed no movement were scored as dead.

Hemolymph volume and intracellular ions. Hemolymph volume was measured using a gravimetric blotting method (20, 24) in n = 16–18 flies per experimental group [i.e., eight groups: two developmental acclimations × two adult acclimations × two treatments (control and following 6 h at 0°C)]. (Control (no cold treatment) flies were individually anesthetized under light CO₂ for 10 s before being quickly weighed to determine wet mass (WM). Flies that received a cold exposure (in chill coma) were taken directly from the cold exposure and weighed without CO₂ anesthetization. Individual flies were then returned to a CO₂-releasing CO₂ canister and placed in the field of view of a dissecting microscope, whereupon fine forceps were used to open the abdomen along the ventral midline. A small triangle of filter paper (0.5 cm²) was placed onto each eye to soak into the paper by capillary action. After 15 s, the paper was removed and the fly was reweighed to determine hemolymph-free wet mass (HFWM). Relative hemolymph mass was calculated as the difference between wet mass (WM) and HFWM, divided by WM (assuming 1 mg = 1 µl).

The same procedure was used to remove hemolymph from a separate set of flies (n = 12 flies per developmental × acclimation temperature group) that were used to quantify intracellular Na⁺ content, without any cold exposure. After hemolymph removal, each fly was weighed and then placed in a 200-µl heat-resistant microcentrifuge tube and dried for 24 h at 60°C in an oven. The dried carcasses were reweighed 24 h later to determine hemolymph-free dry mass (HDFDM). The content of water remaining in the carcass was calculated from the difference between HFWM and HDFDM, and is presumed to be a combination of extracellular water and (to a lesser extent) gut lumen contents and interstitial water, which accounts for <5% of tissue water content in other insects (44, 65).

The Na⁺ content of the dried, hemolymph-free flies were determined using atomic absorption spectrometry. Briefly, 100 µl of nitric acid was added to each dried fly, and samples were left to dissolve with occasional mixing for 48 h at 23°C. Samples were diluted in a final volume of 5 ml with MilliQ water to bring them into the detection limits of the spectrometer (Solaar S4, Thermo Fisher Scientific, Waltham, MA), and average ion concentrations in the carcass were determined by back calculation with the sample-specific dilution factor based on the water content of the fly after hemolymph removal (HFWM-HFDM). The medium fed to the flies was calculated to contain ~1.6 mM Na⁺ (based on the Na⁺ contents of the ingredients), and so intracellular Na⁺ measurements are unlikely to be strongly impacted by the contents of the gut.

Hemolymph ion concentrations. Hemolymph ion concentrations were measured in a different set of animals. Here, hemolymph was sampled from adult flies that were taken either directly from their adult acclimation temperature, or following 6 h at 0°C. The cold exposure was conducted as in the chill coma recovery experiment, except that flies were preloaded into 10-µl pipette tips (sealed with paraflim) to facilitate rapid hemolymph sampling (see below).

Hemolymph was sampled by antennal ablation (41). Briefly, flies were positioned headfirst in a 10-µl pipette tip attached to a tubing system, which applied positive air pressure from behind via a laboratory air supply. The end of the pipette tip was cut to expose the antennae, and one of the antennae was then ablated at its first segment, whereupon a clear droplet of hemolymph flowed out of the wound due to the positive air pressure applied on the body of the fly (41). The pipette tip, with the fly and droplet attached, were immediately detached from the device, and the droplet was deposited into a microvial containing KCl (3 M) to allow for immediate measurement of Na⁺ or K⁺. Sodium and potassium concentrations were measured in separate droplets using ion-selective microelectrodes [note: unlike atomic absorption spectrometry (used to measure intracellular ion concentrations), which quantifies total ion content, ion-selective electrodes quantify ion activity]. Borosilicate glass capillaries [TW-150-4, World Precision Instruments (WPI), Sarasota, FL] were pulled to a tip diameter of ~3 µm with a P-97 Flaming Brown micropipette puller (Sutter Instruments, Novato, CA). The glass micropipettes were silanized at 300°C with *N,N*-dimethyltrimethylsilylamine vapor for 1 h and kept for up to 5 days. Immediately before use, electrodes were either backfilled with the 100 mM of KCl and front-filled with a K⁺ solution.
ionophore (K⁺ ionophore I, cocktail B; Sigma Aldrich, St. Louis, MO) to produce a K⁺-sensitive electrode, or backfilled with 100 mM NaCl and front-filled with an Na⁺ ionophore cocktail [Na⁺ ionophore X; (46)] to produce a Na⁺-sensitive electrode (31). Electrodes were then dipped in a solution of polyvinylchloride (Sigma Aldrich) in tetrahydrofurane (Sigma Aldrich), which prevents capillary rise of the paraffin oil in the electrode (which could otherwise displace the ionophore) (57). Voltage was recorded using an FD223a differential electrometer (WPI) and digitized using a MP100A data acquisition system connected to a computer running AcqKnowledge software (Biopac Systems, Goleta, CA). A glass reference electrode (IB200F-4; WPI) backfilled with 0.5 M KCl was used to complete the circuit. Calibration solutions had a 10-fold difference in the target ion (Na⁺: 15 and 150 mM; K⁺: 10 and 100 mM) with the concentration difference between the two solutions made up with LiCl in both cases. Voltages from the ion-selective electrode were converted to ion concentrations using the following equation:

\[ h = [c] \times 10^{3.5\Delta V} \]

where \([h]\) is the active ion concentration in the hemolymph, \([c]\) is the concentration in one of the calibration solutions, \(\Delta V\) is the voltage difference between the calibration solution and hemolymph, and \(S\) is the slope of the voltage response to the 10-fold concentration difference in the calibration solutions (which should approximately follow a Nernstian relationship of 58 mV). The slopes of Na⁺- and K⁺-selective electrodes used were 56.1 ± 4.0 and 53.0 ± 1.7 mV per 10-fold difference in ion concentration, respectively (means ± SD).

**Hemolymph osmolality.** Hemolymph osmolality was measured using a Clifton nanoliter osmometer (Clifton Technical Physics, Harrison, NY). Flies were taken directly from their adult acclimation conditions (i.e., without cold exposure), and hemolymph was obtained as for the measurement of ion concentrations (above). A pulled borosilicate glass capillary was used to transfer a small sample of hemolymph (~5 nl) from each fly into immersion oil suspended in a platinum disk. The droplets were rapidly frozen, before being slowly warmed until the temperature at which a single small ice crystal remained (the melting point). As each mole of solute decreases the melting point of an aqueous solution by 1.86°C, the melting point was used to determine hemolymph osmolality through reference to standards of known osmolality.

**Data analysis.** All data analysis was done in the R language for statistical computing (v. 3.1.0) (54). The impact of prior thermal environment on chill coma onset temperature (CTmin), CCRT, body mass, hemolymph volume, ion concentration, and osmolality were all analyzed using generalized linear models with developmental and adult acclimation temperature (and cold exposure treatment, where applicable) treated as independent factors. Body mass was a strong predictor of extractable hemolymph volume \((F_{1,135} = 3.7, P < 0.001)\) and was, thus, included as a factor in its analysis. Survival at −2°C was also analyzed by generalized linear models with developmental and adult acclimation temperature treated as factors, but assuming a binomial error distribution and logit link. Exposure times that resulted in 50 and 90% mortality \((L_{50} \text{ and } L_{90}, \text{respectively})\) were calculated from the distribution for each experimental group using the dose.p() function in the MASS package for R (63). When interactions among factors were not statistically significant, general models were rerun with the interaction terms removed, and the model of best fit was chosen by Akaike information criterion. Unless stated otherwise, all summary statistics reported in the text are means ± SE.

**RESULTS**

**The effects of developmental and adult acclimation temperature on chill tolerance.** Developmental and adult acclimation temperature both influenced the CTmin, with flies reared at 15°C \((F_{3,76} = 17.5, P < 0.001)\) or acclimated as adults to 15°C having a significantly lower CTmin \((F_{3,76} = 24.2, P < 0.001; \text{Fig. 3A})\). There was also a significant interaction in the effects of developmental and adult acclimation on the CTmin; the temperature experienced by adults for 6 days had a slightly greater effect on CTmin in flies reared at 15°C \((F_{3,76} = 3.2, P = 0.002)\). By contrast, developmental temperature had no influ-
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By contrast, neither developmental (Fig. 4A) nor adult acclimation to 25°C resulted in a larger Na\(^+\) concentration ratio (Fig. 4B), such that the rank order of the [Na\(^+\)] ratio followed that of the CT\(_{\text{min}}\) and LT\(_{90}\).

As observed for hemolymph [Na\(^+\)], developmental temperature did not significantly affect hemolymph osmolality (F\(_{5,146} = 0.9, P = 0.329\)). Flies acclimated to 15°C as adults, however, had 16% higher hemolymph osmolality (on average) than those acclimated to 25°C (F\(_{5,127} = 3.3, P = 0.003\), prior to any cold exposure (Table 1).

The impacts of chilling on ion and water balance. Although exposure to 0°C led to decreased extracellular [Na\(^+\)] in flies acclimated to 25°C, it had the opposite effect on [Na\(^+\)] in the hemolymph of flies acclimated to 15°C, resulting in a significant interaction in the effects of acclimation temperature and cold exposure treatment on hemolymph [Na\(^+\)] (F\(_{5,146} = 12.3, P = 0.003\); Fig. 5A). By contrast, developmental temperature had little influence on the effects of cold exposure on hemolymph [Na\(^+\)] (F\(_{5,146} = 5.7, P = 0.153\)).

Cold exposure only had a small effect on hemolymph volume in flies acclimated as adults to 15°C, compared with those acclimated to 25°C (a significant interaction of adult acclimation temperature and cold exposure treatment; F\(_{6,139} = 2.1, P = 0.039\); Fig. 5B). Flies acclimated as adults to 25°C lost more than 25% of their hemolymph volume during cold exposure, while those acclimated to 15°C suffered little to no hemolymph volume disruption (Fig. 4B). Flies that developed at 15°C also had significantly greater hemolymph volumes for their body mass than those reared at 25°C prior to cold exposure (F\(_{6,139} = 3.0, P < 0.001\), but adult acclimation temperature had no effect on hemolymph volume prior to cold exposure (F\(_{6,139} = 0.4, P = 0.677\); Fig. 5B).

Neither developmental (F\(_{4,151} = 1.6, P = 0.452\)) nor adult acclimation temperature (F\(_{4,151} = 1.4, P = 0.495\)) had any effect on hemolymph [K\(^+\)] at the acclimation temperature; all flies had hemolymph [K\(^+\)] of \(~17\) mM when measured at their acclimation temperature (Fig. 5C). However, both developmental temperature (F\(_{5,151} = 7.3, P = 0.003\), and the temperature experienced by adults significantly interacted with cold exposure treatment to impact hemolymph [K\(^+\)] (F\(_{5,151} = 16.6, P < 0.001\)). Thus, flies that were reared at or acclimated to 15°C better maintained low (“normal”) K\(^+\) during the 6-h exposure to 0°C (Fig. 5C).

The degree of hemolymph volume disruption (as a proportion of initial volume) correlated with the initial [Na\(^+\)] concentration on CCRT for flies that had spent 6 h at 0°C (F\(_{2,72} = 0.6, P = 0.557\), and instead adult acclimation temperature alone strongly determined CCRT (F\(_{2,72} = 11.0, P < 0.001\), Fig. 3B). Both developmental acclimation (F\(_{4,1468} = 7.7, P < 0.001\)) and adult acclimation (F\(_{4,1468} = 13.8, P < 0.001\)) to 15°C significantly improved rates of survival 24 h following exposure to −2°C, and these treatments also had a significant interactive effect on survival (F\(_{4,1468} = 7.0, P < 0.001\)). This interaction was driven by the survival curves of flies acclimated as adults to 15°C crossing over, such that these two groups shared a similar LT\(_{50}\), despite the flies reared at 15°C having a greater LT\(_{50}\) (Fig. 3, C and D).

The effects of thermal history on Na\(^+\) distribution and hemolymph osmolality. Developmental temperature did not significantly affect hemolymph [Na\(^+\)] (F\(_{5,146} = 3.9, P = 0.168\), but flies acclimated to 15°C as adults had significantly lower hemolymph [Na\(^+\)] prior to any cold exposure than those acclimated to 25°C (~55 mM vs. ~70 mM; F\(_{5,146} = 14.6, P < 0.001\); Fig. 4A). By contrast, neither developmental (F\(_{3,44} = 0.4, P = 0.892\)) nor adult acclimation temperature (F\(_{3,44} = 1.3, P = 0.669\)) impacted intracellular [Na\(^+\)], as flies from all four thermal histories had ~16–18 mM intracellular [Na\(^+\)] (Fig. 4A).

### Table 1. Hemolymph osmolality of adult Drosophila melanogaster females after developmental and adult acclimation to 15°C or 25°C

<table>
<thead>
<tr>
<th>Developmental Temperature, °C</th>
<th>Adult Acclimation Temperature, °C</th>
<th>n</th>
<th>Hemolymph Osmolality, mosmol kgH(_2)O</th>
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<tr>
<td>15</td>
<td>15</td>
<td>7</td>
<td>456 ± 30</td>
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<tr>
<td>15</td>
<td>25</td>
<td>8</td>
<td>404 ± 15</td>
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<tr>
<td>25</td>
<td>15</td>
<td>6</td>
<td>449 ± 15</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>7</td>
<td>375 ± 11</td>
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Values are expressed as means ± SE. Flies acclimated to 15°C as adults had higher hemolymph osmolality than those acclimated to 25°C, while developmental temperature had no effect on hemolymph osmolality of adult flies (see text for details).
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**DISCUSSION**

The additive effects of developmental temperature and adult acclimation on chilling tolerance. To characterize the chill tolerance of D. melanogaster females, we measured the critical thermal minimum (CTmin), chill coma recovery time (CCRT) after 6 h at 0°C, and survival 24 h after exposure to −2°C for varying lengths of time. Low-temperature tolerance is highly plastic in D. melanogaster (14, 56), and in the present study, thermal history had a strong impact on chill tolerance, regardless of the measure used. However, the effects of developmental and adult acclimation temperatures on chill tolerance somewhat differed depending on the measure of cold tolerance used. This agrees with repeated observations that these common measures often do not tightly correlate within or among Drosophila species (3, 56), and results from locusts also suggest that different measures (particularly, the CTmin and CCRT) are associated with different physiological mechanisms (18, 40). Regardless of the measure of chill tolerance used, however, the thermal environment experienced for 6 days as an adult had a greater effect on chill tolerance than the temperature at which the flies developed. Moreover, the effects of thermal acclimation on the CTmin and lethal time at low temperature were additive (developmental temperature had a persistent effect on the thermal tolerance of adults). As previously reported by Colinet and Hoffmann (14), we found no evidence that CCRT following a cold stress is modified by developmental temperature in D. melanogaster. Flies that shared an adult acclimation temperature had relatively indistinguishable recovery times following a 6-h exposure to 0°C.

Chill-tolerant flies maintain low extracellular [Na⁺]. To characterize the Na⁺ distribution of D. melanogaster raised in different thermal environments, we measured both extracellular and intracellular [Na⁺]. Because extracellular [Na⁺] varied among the experimental groups and intracellular [Na⁺] did not, the [Na⁺] ratio calculated from the mean [Na⁺] of the extracellular and intracellular fluids was markedly different depending on the thermal environment that the flies experienced. The rank order of chill tolerance matched that of hemolymph [Na⁺] among the four groups prior to any cold exposure. Thus, both developmental temperature and adult acclimation temperature appear to impact the distribution of Na⁺ between the hemolymph and intracellular space or gut lumen. As for the chill tolerance measures, the effects of long-term adult acclimation temperature (i.e., the more recent thermal history) on Na⁺ distribution were stronger than those of developmental temperature. These observations are consistent with previous comparative measures showing that Drosophila species that are more cold-tolerant also maintain low extracellular [Na⁺], even when reared under common conditions (39). Therefore, it seems that reductions in the ratio of extracellular to intracellular [Na⁺] may be a common consequence of developmental and adult cold acclimation, as well as the evolution of chilling tolerance within the Drosophila genus. We note that changes to the Na⁺ concentration ratio may not always occur through reductions in hemolymph [Na⁺]; cold acclimation induces an increase in extracellular [Na⁺] in tropical cockroaches, but a greater proportional increase in intracellular [Na⁺] of the muscle, which still results in an overall reduction in the Na⁺ distribution ratio and depolarization of the Na⁺ equilibrium potential (35).

Flies with a low hemolymph [Na⁺] defend hemolymph volume and [K⁺] at low temperatures. To test the hypothesis that the observed reductions in the ratio of extracellular-to-intracellular [Na⁺] protect against a loss of hemolymph water and an associated rise in hemolymph [K⁺], we measured...
hemolymph volume, [Na\(^+\)], and [K\(^+\)] prior to and following a 6-h exposure to 0°C.

Chill-tolerant flies (those that were reared at or acclimated to 15°C) better maintained low extracellular K\(^+\) during a 6-h exposure to 0°C. By contrast, those flies that developed or spent their adult life at 25°C had high hemolymph [K\(^+\)] following the same cold exposure. As observed with all three estimates of chilling tolerance, adult acclimation temperature more strongly determined the extent of hemolymph K\(^+\) disruption (\(\Delta[K^+]\)) than developmental acclimation. Thus, the loss of hemolymph volume appears to concentrate K\(^+\) in the hemocoel, and this concentration is more pronounced in the acclimation groups that are less cold-tolerant. The degree of [K\(^+\)] increase is larger than would be expected by the hemolymph volume decrease alone, which suggests that some [K\(^+\)] also leaks down its concentration gradient from the intracellular spaces or gut lumen into the hemolymph during chilling, as has also been observed in chill-susceptible locusts (2, 17). These findings are, therefore, in support of the current model of cold-induced ion and water balance disruption (Fig. 1) and add to a growing body of evidence from diverse insect species (including blattodean, orthopteran, hemipteran, and dipteran representatives) that the ability to maintain low extracellular [K\(^+\)] is closely correlated with chilling tolerance (2, 13, 17, 34, 35, 39, 43, 44).

Chill-tolerant flies do not only avoid Na\(^+\) leak in the cold but may also counteract the effects of low temperature on hemolymph [Na\(^+\)]. The most tolerant flies (those acclimated as adults to 15°C) appear able to reverse the effect of cold exposure on hemolymph [Na\(^+\)] in the cold, such that extracellular [Na\(^+\)] was higher following cold exposure. This seemingly adaptive response may be mediated by activation of Na\(^+/\)H\(^+\) transport rates in the cold, or via an induced reduction in Na\(^+\) permeability at low temperatures (42), either of which would serve to maintain [Na\(^+\)] in the hemolymph, and thereby contribute to the maintenance of water balance.

What molecules replace ions in the hemolymph? Both within- (present study) and among-species (39) variation in Drosophila chill tolerance has been correlated specifically with reduced extracellular [Na\(^+\)]. Such a change could “decouple” water balance from Na\(^+\) balance and protect against hemolymph volume disruption in the cold (Fig. 1). However, this response would only be seen if reductions in [Na\(^+\)] at the acclimation conditions are matched or exceeded by the accumulation of other organic or inorganic osmolytes in the hemolymph (39). Although flies acclimated to 15°C as adults had lower extracellular [Na\(^+\)] than those acclimated to 25°C, they had higher hemolymph osmolality, which suggests that extracellular [Na\(^+\)] reductions are exceeded by the accumulation of other compatible osmolytes.

The accumulation of high extracellular concentrations of cryoprotectants (which are also osmolytes) is a hallmark of the specialized cold tolerance strategies of freeze tolerance and freeze avoidance (38). By contrast, relatively small increases in the whole body concentrations of osmolytes, including trehalose, sorbitol, glycerol, and glucose have been noted in concert with increased chill tolerance of chill-susceptible insects, including D. melanogaster (33, 36, 37, 47, 53, 66). These changes have been too small in magnitude to afford cryoprotection through their colligative effects (37, 52), yet it is well known that such compounds also have noncolligative effects [e.g., protection against membrane phase transitions (10) or protein denaturation (11)]. Thus, our data support the hypothesis that small increases in compatible osmolytes complement the decrease in extracellular [Na\(^+\)] found in cold-acclimated flies, such that hemolymph osmolality is maintained (or increased), while water balance becomes less dependent on the distribution of small, permute ions, such as Na\(^+\) (39) (Fig. 1).

**Perspectives and Significance**

Exposure to low temperatures during development or adult life can have strong impacts on the thermal tolerance and overwintering success of insects. In recent years, several studies have highlighted a loss of ion and water homeostasis as a primary cause of injury in insects that are susceptible to mild chilling. In this study, we tested the current model of ion and water balance disruption in chill-susceptible insects in Drosophila melanogaster, and simultaneously examined whether developmental and adult plasticity in chilling tolerance are driven by improvements in the ability to maintain ion balance at low temperatures. We demonstrated that chill-sensitive flies have high initial hemolymph Na\(^+\)\(^–\), lose hemolymph volume during chilling, and develop damagingly high concentrations of extracellular K\(^+\). By contrast, chill-tolerant flies have low extracellular Na\(^+\) concentrations, and defend their hemolymph volume and K\(^+\) concentration in the cold. These findings support the current model of ion and water balance disruption and extend the earlier evidence on which this model was built to the most ubiquitous model insect.

The combination of hemolymph-sampling techniques for small insects, such as those used herein (41) and a wealth of
genetic and molecular tools available for D. melanogaster, allows for focused study on the molecular mechanisms underlying the apparent ionoregulatory differences associated with chill tolerance and chilling injury in insects. Thus, further study should, for example, draw on current knowledge of insect renal function (7, 12, 49) to determine how endocrine control of ion and water balance may underlie thermal acclimation and adaptation. We expect such integrative, hypothesis-driven studies will ultimately uncover novel candidate genes and proteins regulating insect thermal tolerance and may also give insights into the evolutionary origin of the cryoprotectant accumulation that is a hallmark of freeze tolerance and freeze avoidance.

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AUTHOR CONTRIBUTIONS

Author contributions: H.A.M, V.L. and J.O. designed and conceived the research; H.A.M and J.L.A. performed the experiments; H.A.M. described and analysed the data; H.A.M. and J.O. drafted the manuscript; and all authors revised the manuscript.

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